J Mol Evol (2000) 51:110–121
DOI: 10.1007/s002390010071

JOURNAL OF MOLECULAR EVOLUTION

Six Opsins from the Butterfly *Papilio glaucus:* Molecular Phylogenetic Evidence for Paralogous Origins of Red-Sensitive Visual Pigments in Insects

Adriana D. Briscoe

Harvard University, Department of Organismic and Evolutionary Biology, Cambridge, MA 02138, USA

Received: 6 December 1999 / Accepted: 3 April 2000

Abstract. It has been hypothesized that the UV-, blue-, and green-sensitive visual pigments of insects were present in the common ancestor of crustaceans and insects, whereas red-sensitive visual pigments evolved later as a result of convergent evolution. This hypothesis is examined with respect to the placement of six opsins from the swallowtail butterfly *Papilio glaucus* (PglRh1–6) in relationship to 46 other insect, crustacean, and chelicerate opsin sequences. All basal relationships established with maximum parsimony analysis except two are present in the distance and maximum likelihood analyses. In all analyses, the six *P. glaucus* opsins fall into three well-supported clades, comprised, respectively, of ultraviolet (UV), blue, and long-wavelength (LW) pigments, which appear to predate the radiation of the insects. Lepidopteran green- and red-sensitive visual pigments form a monophyletic clade, which lends support to the hypothesis from comparative physiological studies that red-sensitive visual pigments in insects have paralogous origins. Polymorphic amino acid sites (180, 197, 277, 285, 308), which are essential for generating the spectral diversity among the vertebrate red- and green-sensitive pigments are notably invariant in the *Papilio* red- and green-sensitive pigments. Other major tuning sites must be sought to explain the spectral diversification among these and other insect visual pigments.

Key words: Evolution — Lepidoptera — Cones — Vertebrates — Color vision — Opsin phylogeny — Swallowtail — Papilionidae

Introduction

Insect compound eyes are composed, minimally, of three distinct classes of photoreceptor cells, with peak sensitivities clustered around 350 nm (UV), 440 nm (blue), and 520 nm (green) (Chittka 1996). The few exceptions to this visual bauplan include the loss of the blue-sensitive photoreceptor cells in some Dictyoptera (Mote and Goldsmith 1970) and Hymenoptera (Chittka 1996), the gain of additional short-wavelength photoreceptor cells in Odonata (Yang and Osorio 1991) and Diptera (Montell et al. 1987), and the appearance of red-sensitive photoreceptor cells in some species of Lepidoptera (Bernard 1979), Hymenoptera (Peitsch et al. 1992), and Odonata (Meinertzhagen et al. 1983). Exceptional classes of photoreceptor cells within insects, such as the red-sensitive photoreceptor cells, pose a puzzle because of their sporadic appearance. Based on physiology alone, it is not clear whether the visual pigments expressed within those cells have a single origin or are the result of convergent evolution. This study examines the origins of red-sensitive photoreceptor cells in insects by characterizing the opsins from the swallowtail butterfly *Papilio glaucus* and examining their relationship other insect opsins.

Present address: Department of Molecular and Cellular Biology, 1007 E. Lowell/Life Sciences South 444, University of Arizona, Tucson, AZ 85721, USA

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession numbers AF077189 (PglRh1), AF077190 (PglRh2), AF067080 (PglRh3), AF077193 (PglRh4), AF077191 (PglRh5), and AF077192 (PglRh6).

Visual pigments are composed of an opsin protein, a member of the G protein–coupled receptor family and a light-sensitive chromophore derived from retinal. In the eye visual pigments detect light, information which is conveyed to the nervous system by a chain of biochemical events that begins with visual pigment photoactivation. Because most organisms synthesize a single chromophore, the diversity of visual pigment absorption spectra on which vision is based, depends primarily on the opsin amino acids lining the chromophore binding pocket. Selection for amino acid substitutions at these key sites has led to the spectrally diverse array of visual pigments present in different classes of photoreceptor cells.

Butterflies of the genus *Papilio* have four spectrally distinct classes of photoreceptor cells in their retina, with peak sensitivities in the UV (360 nm), blue (450 nm), green (520 nm), and red (575 nm) (Horridge et al. 1983; Arikawa et al. 1987, 1999a). Peak spectral sensitivities of photoreceptor cells are generally thought to conform closely to the absorption spectrum maximum (λmax) of the visual pigments expressed within those cells, although they can be modified due to the filtering effects of screening pigments (Gribakin 1988) among other reasons. Indeed, in *Papilio xuthus*, a violet (390 nm) class of photoreceptor cell has also been identified, and is thought to be the result of the interaction between a co-expressed UV-absorbing filtering pigment and a UV-sensitive[1] visual pigment (Arikawa et al. 1999b). Because in most organisms, only a single opsin transcript is found per photoreceptor cell (but see Sakamoto et al. 1996; Kitamoto et al. 1998), only four distinct opsin transcripts were expected to be found in the *Papilio* retina.

Instead, I isolated six distinct opsin-encoding *Papilio glaucus* cDNAs from head tissue mRNA, three of which were novel, and three of which (PglRh1–3) were homologous to LW opsins (PxRh1–3) reported by Kitamoto et al. (1998) from *P. xuthus.* The three additional *P. glaucus* opsin sequences include a homologue of other insect UV (PglRh5) and blue (PglRh6) pigments, and a new *P. glaucus* LW pigment (PglRh4), unexpected from electrophysiological studies of the *Papilio* retina. By analyzing the relationship between these *Papilio* opsins and other known invertebrate opsins, I provide molecular phylogenetic evidence in support of a comparative physiological hypothesis that red pigments have paralogous origins in insects. Because gene conversion has occurred often between the red and green pigment genes during higher primate evolution (Zhou and Li 1996), I also sequenced an intron from the LW *Papilio* opsins to explore this possibility in *Papilio.*

By examining the five amino acid residues known to generate the largest spectral differences between the vertebrate red and green pigments, I provide evidence that spectral diversification of the four *P. glaucus* LW (PglRh1–4) pigments does not occur through substitutions at these critical amino acid sites. This leads to the intriguing observation that although natural selection has occurred in both vertebrates and arthropods for multiple long wavelength pigments in the same organism, the major tuning sites used to generate their spectral variation may be different.

Materials and Methods

RNA Isolation and cDNA Synthesis. A female *P. glaucus* pupa, purchased from Ward's and reared in the lab until eclosure, was snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from the frozen head wtih Trizol (GibcoBRL), and resuspended in 100 μl DEPC-treated H_2O. For 3′RACE, sscDNA was synthesized by combining 10 μl total RNA, SuperScript Rnase H-reverse transcriptase (GibcoBRL), and a 35-bp adaptor primer (Frohman et al. 1988). The Marathon cDNA kit (Clontech) was used to synthesize 80 μl of dscDNA from another aliquot of total RNA for 5′RACE and PCR of full-length clones.

3′ and 5′ RACE, Cloning and Sequencing. Partial sequences (324 bp in length) obtained from 3′RACE with the degenerate primer GSP1 (Fig. 1) were previously reported (Briscoe 1998, 1999). To obtain additional sequence for phylogenetic analysis, 5′RACE products were generated by amplifying dscDNA with gene-specific reverse primers (Table 1) and an adaptor primer (PCR conditions: 1 min 94°C, then 30 cycles of 30 s at 94°C, 30 s at 55–60°C, 3 min at 68°C). To verify the sequences, gene-specific primers spanning the coding region were used to amplify a third set of PCR products from dscDNA using the Advantage cDNA PCR kit (Clontech) (PCR conditions: 1 min 94°C, then 30 cycles of 30 s at 94°C, 3 min at 62°C). PCR products were incubated with Taq DNA polymerase (Promega), purified (Geneclean II kit, Bio 101), cloned into pCR 2.1 (Original TA cloning, Invitrogen) or pCR2.1-TOPO (TOPO TA cloning, Invitrogen) plasmids and sequenced on an ABI370A Automated DNA sequencer with Dye primer or Dye deoxy terminator kits (Perkin Elmer). For each gene, 3–8 clones from the 3′ RACE reaction, 3–4 clones from the 5′ RACE reaction, and 2–4 clones spanning the coding region were sequenced in both directions.

Isolation of Genomic DNA. Genomic DNA was isolated as previously described (Briscoe 1999). Gene-specific primers were designed based on regions of exon 7 and exon 8 (relative to PglRh3). The primers used to amplify this genomic fragment from PglRh1 (Rh1F/Rh1R) and from PglRh4 (Rh4F/Rh4R) are given in Table 1. The PCR products were amplified, cloned, and sequenced as described above.

Northern Blot Analysis. The optic lobe or brain of some insects contains extraretinal photoreceptors (Felisberti and Ventura 1996; Schulz et al. 1984; Fleissner et al. 1993; Hofbauer and Buchner 1989). To guard against the possibility that the *P. glaucus* opsins are found in the optic lobe or brain and not in the retina, the expression of opsin mRNA was examined by Northern blot analysis. Total RNA was isolated from the thorax and retinas of a male *P. glaucus*. dsDNA probes

[1] Abbreviations: Red pigment = red-sensitive visual pigment; green pigment = green-sensitive visual pigment; blue = blue-sensitive visual pigment; UV pigment = UV-sensitive visual pigment.

Fig. 1. Alignment of six *Papilio glaucus* opsin (PglRh1–6) sequences. *Brackets:* indicate the boundaries of seven membrane-spanning helices as inferred by the Predict-Protein PHDhtm program (www.embl-heidelberg.de/predictprotein/) (Rost et al. 1995). Two putative start codons were identified in PglRh5 (See 5′UTR in Fig. 2). The longer amino acid sequence in shown. The location of the primer used to generate the 3′RACE products, GSP1 (5′GANCARGCNA-ARAARATGA 3′), is marked by a *black arrow.* Amino acid sites homologous to those critical to spectral tuning in human red- and green-sensitive visual pigments are underlined (see Table 3).

Table 1. Gene-specific opsin primers

Gene	Primer	Sequence 5′ to 3′
PglRh1	P1R3	AGGTCAAGGTAGAAGAGG
	Rh1F	ATGAGGGAGCAAGCAAAGAA
	Rh1R	AAGACCGAACCCCAAATC
PglRh2	P2R3	GGTGTGGCTGACGAGGTGT
PglRh3	P3R1	TGTATTTCGGGTGGCTGAT
PglRh4	P4R1	GTAAGCAATCCGTATCAC
	Rh4F	CTAAGATCATCGGAGTCCGC
	Rh4R	GAGAGAGCCCCAAATGGTAGTC
PglRh5	P5R1	GTATTTCGGATGACTAATGG
PglRh6	P6R2	GTGGCAACAGGCGACAGC

were prepared from gel-purified PCR products in random primer labeling reactions (Random Primer Fluorescein Labeling kit, DuPont). Blot preparation, hybridization (overnight at 60°C), and washing was performed according to the NorthernMax-Gly (Ambion) instructions. Blocking, antibody incubation, and chemiluminescence substrate incubation proceeded according to the Random Primer labeling kit (DuPont) protocol. The RNA molecular weight marker (Boehringer Mannheim) was visualized by staining 3 min in 0.02% methylene blue, 0.3 *M* NaOAc (pH 5.5), and destaining 1 min in water (Herrin and Schmidt 1988).

Phylogenetic Analysis. A BLAST search of GenBank, and a Medline literature search produced 54 invertebrate opsin sequences. Amino acid sequences were aligned in Clustal W (Thompson et al. 1994), adjusted by eye in SeqPup (version 0.6), and then used as a guide in the alignment of the nucleotide sequences. The rate of synonymous substitution at first, second, and third positions was calculated to evaluate whether nucleotide or amino acid analysis would be more appropriate. When uncorrected distances were calculated for third positions in PAUP*, 19% of pairwise comparisons had distances greater than 70%, suggesting saturation for many comparisons. Calculation of the proportion of synonymous substitution between the *Papilio* opsins in MEGA (Kumar et al. 1993) confirmed this suggestion, with all LW (Rh1–4) pairwise comparisons showing saturation (Table 2). Similarly, the number of substitutions at third positions evaluated over a preliminary parsimony tree in MacClade was 2.5 and 4.1 times higher, respectively, than first and second positions. The consistency index (CI) for first, second, and third positions was 0.40, 0.51, and 0.24, with 1 indicating no homoplasy.

Because maximum parsimony can be mislead by high levels of homoplasy, and because for some of the taxa, only amino acid sequences were available, the more thorough parsimony searches were restricted to the larger amino acid data matrix. Maximum parsimony analysis of the amino acid matrix (343 sites corresponding to aa 26–356 in PglRh1) was implemented in PAUP*4d64 (Swofford 1998), with a step matrix derived from a data set of 125 G protein–coupled receptors (Rice 1994) (200 bootstrap replications, random-sequence addition, and TBR branch swapping). Distance analysis of the amino acid matrix used minimum evolution as the objective function with mean character difference as the distance measure. Bootstrap support was calculated via a heuristic search, 500 bootstrap replicates, and TBR branch swapping.

A smaller matrix of 28 nucleotide sequences was also analyzed using maximum likelihood (981 sites corresponding to bp 199 to 1,173 in PglRh1). To shorten the length of time required for tree searches, maximum parsimony analysis was first conducted on this smaller matrix assuming unordered characters and equal weighting of all nucleotide sites (heuristic search, 500 random addition sequence replicates, TBR branch swapping). Maximum likelihood scores were then calcu-

Table 2. Uncorrected proportion of synonymous (below diagonal) and nonsynonymous (above diagonal) differences

	PglRh1	PglRh2	PglRh3	PglRh4	PglRh5	PglRh6	PxRh1	PxRh2	PxRh3
PglRh1	—	0.11	0.11	0.13	0.47	0.45	0.02	0.11	0.11
PglRh2	0.83	—	0.12	0.11	0.47	0.47	0.11	0.02	0.12
PglRh3	0.76	0.74	—	0.14	0.47	0.46	0.11	0.12	0.01
PglRh4	0.83	0.74	0.81	—	0.46	0.47	0.12	0.11	0.15
PglRh5	0.47	0.47	0.47	0.76	—	0.37	0.48	0.46	0.48
PglRh6	0.45	0.47	0.46	0.74	0.36	—	0.45	0.47	0.46
PxRh1	0.47	0.77	0.74	0.80	0.48	0.45	—	0.11	0.11
PxRh2	0.81	0.35	0.74	0.78	0.46	0.47	0.74	—	0.12
PxRh3	0.73	0.74	0.32	0.81	0.48	0.46	0.73	0.74	—

lated for the single resulting parsimony tree, under four models: Jukes-Cantor, F81, HKY, and GTR (Maddison et al. 1999). The GTR + %I + Γ model fit the data better than the less complex HKY + %I + Γ model ($p < 0.005$, likelihood ratio test statistic = 17.7367, four degrees of freedom). Maximum likelihood estimates of the proportion of invariable sites, the shape parameter of the gamma distribution, and the substitution rate matrix were calculated from the parsimony tree and used as starting assumptions for maximum likelihood analysis (heuristic search, TBR branch swapping, empirical base frequencies).

Results

Isolation of Six Papilio glaucus *Opsin cDNAs*

3′RACE produced two major bands of approximately 700 bp and 800 bp each, which were gel-purified, cloned, and sequenced. Fifty sequenced cones of the 3′RACE products resulted in six opsin-encoding fragments, 696 bp, 826 bp, 809 bp, 508 bp, 720 bp, and 717 bp long, which were designated PglRh1–6, respectively (note: these names do not imply any homology with the *Drosophila* Rh1–6 opsins). These sequences were used to design six gene-specific reverse primers (Table 1) used to amplify the remaining coding regions and 5′UTRs. Each gene specific primer pair amplified single unique opsin-encoding products. The sequenced 5′RACE products overlapped with the 3′RACE products, and without ambiguity matched the 3′RACE sequences. A third set of cloned PCR products spanning the coding region of five of the opsins, generated with gene-specific primers located in the 5′ and 3′UTRs, also yielded single transcripts, which matched the 3′ and 5′RACE sequences.

The deduced amino acid sequences of the six *P. glaucus* opsins are shown (Fig. 1). Including the 5′ and 3′UTRs, a total of 1,585 bp of PglRh1, 1,747 bp of PglRh2, 1,679 bp of PglRh3, 1,159 bp of PglRh4, 1,526 bp of PglRh5, and 1,638 bp of PglRh6 were cloned and sequenced in both directions. Four of the cDNAs encode opsins varying in length from 379 (PglRh1 and PglRh3), 380 (PglRh2), to 381 (PglRh6) amino acids. The cDNA encoding PglRh5 has two possible start codons, the second of which contains a putative translation initiation sequence CCGCGAUGG (Fig. 2) that differs from the optimal mammalian sequence CC(A/G)CCAUGG (Kozak 1991) by a single nucleotide and encodes a 375-aa protein. The first putative start codon, which is 18 bp upstream, is not found within an optimal translation initiation sequence and encodes a longer protein product (381 aa). The longer of the two putative protein sequences is shown (Figs. 1 and 2). The start codon and an estimated 104 bp of PglRh4 coding region were not isolated due possibly to premature termination of reverse-transcription, or low abundance of full length RNA (see expression analysis results below).

Isolation of an Intron from PglRh1 and Pg1Rh4

Elsewhere I have reported the genomic sequence of PglRh3 (Briscoe 1999). PglRh3 has at least eight introns, one of which, intron 7, is 78 bp in length (Briscoe 1999). An intron with splice sites identical to intron 7 of PglRh3 (between bp 952–953) was isolated from PglRh1 and PglRh4. The intron from PglRh1 was 264 bp long, and the intron from PglRh4 was 225 bp.

mRNA Expression of the P. glaucus *Opsins*

DNA probes of PglRh1, PglRh2, PglRh5, and PglRh6 cDNAs, hybridized to total RNA from retina and thorax tissue, revealed single bands in the retina lane (Fig. 3). No visible bands were present in the thorax lane. Several attempts at detecting *P. glaucus* PglRh3 and PglRh4 mRNA were unsuccessful, perhaps due to the low quantity of transcript present in our sample. The low abundance of PglRh4 transcript was especially evident in the RT-PCR experiments, in which PglRh4 amplified much more weakly than the other *Papilio* opsins (1 out of 50 sequenced clones). By comparison, amplification of a 762-bp PglRh4 genomic fragment was comparable to the amplification of genomic fragments of PglRh1–3 with primers from the same region (data not shown). Kitamoto et al. (1998) report that cRNA probes of PxRh1–3, orthologues of PglRh1–3 (see Fig. 4), hybridize to photoreceptor cells in the retina. These results collectively indicate that PglRh1–3, 5, and 6 have a visual function.

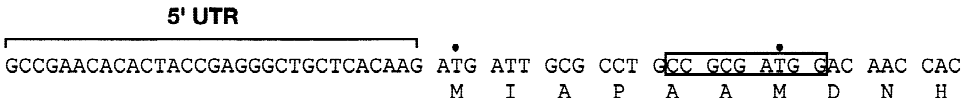

Fig. 2. Nucleotide sequence of the first 60 bp of cloned *PglRh5* cDNA. *Box:* sequence that resembles the vertebrate consensus transcription initiation start site (Kozak 1991). *Black dots:* Location of two putative start codons.

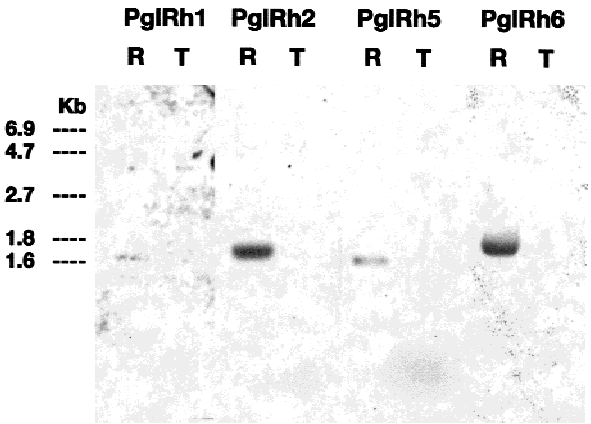

Fig. 3. mRNA expression of *Papilio glaucus PglRh1–6* opsin genes. Northern blot analysis using six separate clones encoding PglRh1–6 revealed the *PglRh1, PglRh2, PglRh5,* and *PglRh6* genes are transcribed as mRNAs that fall between 1.5 and 1.8 kb in length. These transcripts were present in the retina but not the thorax of a male *P. glaucus* specimen. A separate filter with total RNA from both the retina and thorax of this male was probe with randomly primed DNA complementary to *P. glaucus* 18s rRNA, as a control for the presence of RNA in each lane. *PglRh3* and *PglRh4* were not present at detectable levels using this method (data not shown).

PglRh4 may or may not have a visual function, which spatial expression analysis in *P. glaucus* will clarify (Briscoe and Nagy in preparation).

Phylogenetic Relationships of Papilio *Opsins*

Bootstrap trees recovered by maximum parsimony and minimum evolution on the large (54-taxa) amino acid data set were identical everywhere, except in three respects. In the minimum evolution tree, *D. melanogaster* Rh6 was no longer clustered with *Limulus,* but became basal to all insect LW opsins. The ant–honey bee LW clade collapsed into a basal polytomy, and the relationships within the *Drosophila* Rh1 and Rh2 clades were rearranged. The maximum likelihood analysis of the nucleotide sequences of 28 taxa recovered the same branching order of the deepest nodes recovered in the maximum parsimony analysis, except in two places. Like the minimum evolution analysis, maximum likelihood analysis placed *D. melanogaster* Rh6 with the other insect LW opsins. Also, the *Manduca sexta* 1 opsin was clustered with PglRh4 in the maximum likelihood analysis, unlike the parsimony analysis, where the *Manduca* LW opsin was clustered with PglRh2.

PglRh1–6 fall into three major clades in all analyses (Fig. 4). PglRh5 falls within an UV clade and is most closely related to the moth opsin *M. sexta* 2 with 100% parsimony bootstrap support. Other members of the clade include the *Drosophila* Rh3, Rh4, and honey bee UV opsins. Within the blue clade, PglRh6 and *M. sexta* 3 sequences are grouped (100% bootstrap support) and are closely related to the honey bee (93% bootstrap support) and *Drosophila* blue opsins (86% bootstrap support) (Bellingham et al. 1997; Townson et al. 1998; Salcedo et al. 1999). PglRh5 and PglRh6 are the first UV and blue opsin homologues cloned from butterflies.

Monophyly of Lepidopteran LW Opsins

Previously, I reported that PglRh3 is a member of the LW clade and that its shared intron splice sites independently support the deeper clustering of the LW clade with a clade of blue–green opsins in *Drosophila* (Rh1 and Rh2) (Briscoe 1999). Of the six *P. glaucus* opsins reported so far, PglRh3 is most closely related to PglRh1 (100% bootstrap support). PglRh2 and PglRh4, in that order, are basal to the PglRh1–PglRh3 clade. The movement of *M. sexta* 1 from the clade that includes PglRh2–PxRh2 to form a new clade with PglRh4 suggests two possible lepidopteran ancestral states. Either the ancestor to moths and butterflies already had two LW genes or the ancestor to moths and butterflies had only one.

Discussion

No Evidence for Recent Gene Conversion Among the Papilio *LW Opsins*

Gene conversion is a process by which duplicated genes become homogenized through unequal crossing-over (Li 1997). Gene conversion has been found, for instance, among the primate red and green pigments (Zhou and Li 1996). Because recent gene conversion is expected to obscure phylogenetic relationships, I examined this possibility among the *Papilio* LW opsins. Table 2 gives the uncorrected (p-distance) proportion of synonymous and nonsynonymous differences between the *P. glaucus* and *P. xuthus* opsins. The proportion of synonymous substitutions between the different LW (Rh1–4) opsins within a species is extremely high (0.74–0.83 in *P. glaucus* and 0.72–0.74 in *P. xuthus*), whereas the proportion of syn-

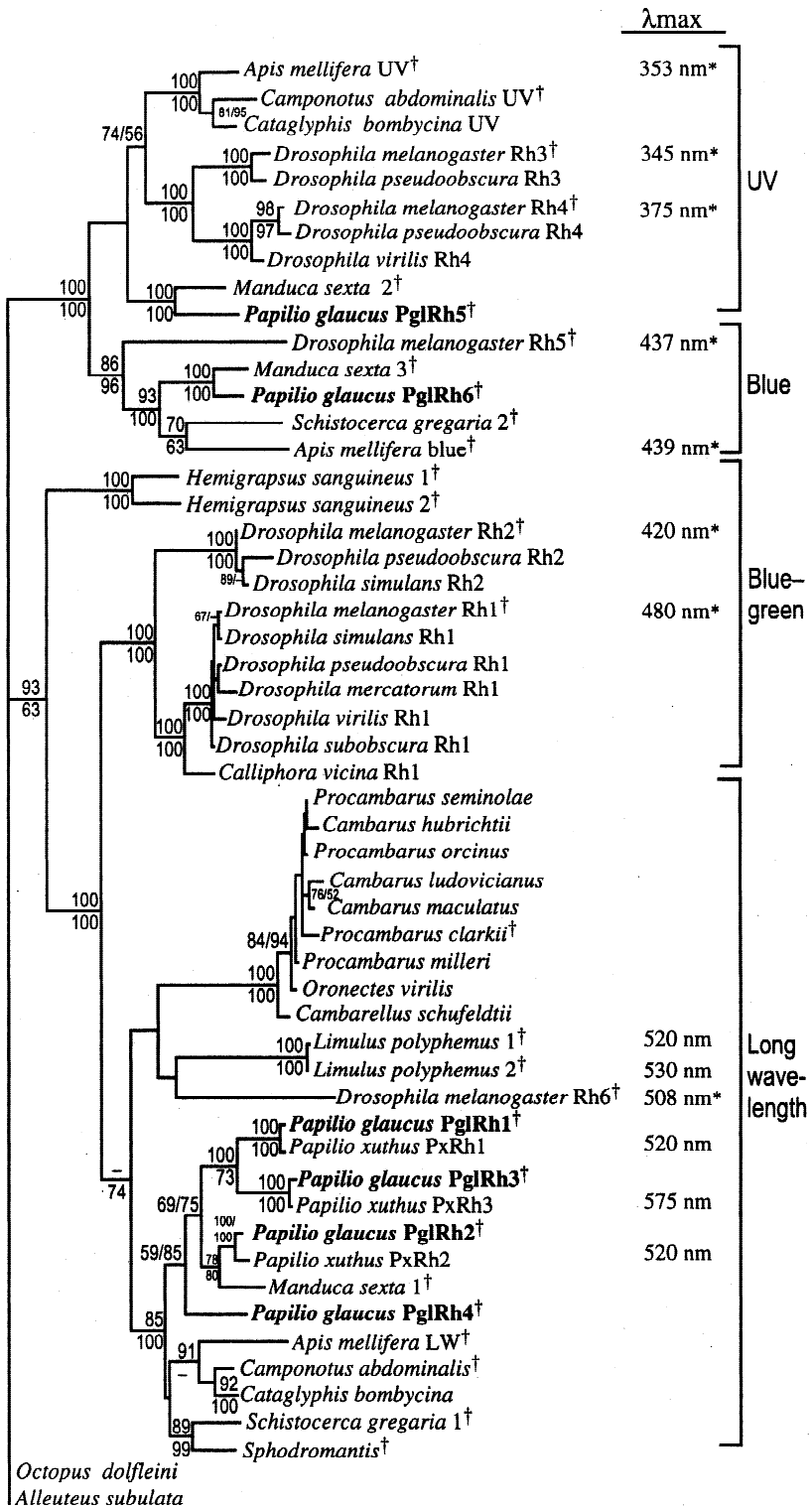

Fig. 4. One of 15 equally parsimonous trees from a heuristic search of opsin amino acids sequences using a step-matrix (informative sites = 302, tree length = 14,530, Consistency Index = 0.57, Retention Index = 0.83). The 15 equally parsimonious trees differed in branching order only within the clade of crayfish opsins and within the clade of *Drosophila* Rh1 opsins. Bootstrap values *above* the nodes are from a parsimony analysis (250 replicates) and bootstrap values *below* the nodes are from a separate distance analysis (500 replicates). Dashes indicate bootstrap support <50%. Taxa included in the maximum likelihoood analysis are indicated with a *cross*. λmax values of opsins that have been transgenically expressed in *Drosophila melanogaster* (*asterix*) or whose mRNA expression patterns have been correlated with electrophysiologically determined spectral sensitivities are shown. *Limulus* data are from Smith et al. (1993), *D. melanogaster* data from Zuker et al. (1988) and Feiler et al. (1992). *Apis* data are from Townson et al. (1998), *Hemigrapsus* from Sakamoto et al. (1996), and *Papilio* data from Bandai et al. (1992) and Kitamoto et al. (1998).

onymous substitutions between orthologs in different species is much lower (0.32–0.47). This suggests that synonymous sites are not currently being homogenized by gene conversion.

Another hallmark of gene conversion is that recently converted genes may have long stretches of DNA with little or no substitutions. After aligning all pairwise combinations of LW opsins, I counted the length of identical sequence between them. The longest identical stretch of shared sequence without substitutions was 20 bp in length and was between PglRh2 and PglRh3, which are not the most similar of the four opsins. Due to gene conversion, the introns of the primate red and green pigments have little to no variation between genes, whereas

the coding region has considerably higher variation because of natural selection (Zhou and Li 1996). In the case of the three introns I examined from PglRh1, PglRh3, and PglRh4, there was considerable variation in length (78–264 bp) and enough sequence divergence that they were impossible to align. Similarly, the 3′ and 5′ ends of these genes could not easily be aligned. Although there is no way to rule out gene conversion early in the history of *Papilio* LW opsin evolution, a recent gene conversion event seems unlikely.

Origins of UV, Blue, and Green Pigments in Insects

Insect compound eyes are typically composed (see Mote and Goldsmith 1970) of at least three distinct classes of photoreceptor cells, with peak sensitivities clustered around 350 nm (UV), 440 nm (blue), and 520 nm (green), which are hypothesized to have arisen approximately 500 million years ago (Chittka 1996). This conclusion, which is drawn from the mapping of electrophysiological data on a species phylogeny of chelicerate, crustacean, and insect taxa, is largely supported by the overall branching pattern of the opsin gene tree that is divided so far into three major clades (Fig. 4). The average λmax values of pigments within these clades is 358 nm (UV clade), 439 nm (blue clade), 533 nm (LW), respectively—averages that closely match Chittka's averages derived, with one exception, from an independent set of species. While the λmax values within clades are within 8–55 nm of each other, the difference in average λmax value between clades is 81 (UV–blue) to 94 nm (blue–LW).

Although only a few chelicerate and crustacean sequences are available, the opsin phylogeny supports the hypothesis from comparative physiology (Chittka 1996) that the LW pigments have an early origin that traces back to the ancestor of the chelicerates and crustaceans. No chelicerate or crustacean UV opsins have yet been characterized, so the age of the UV clade remains unclear. Within the insects, however, the UV and blue clades appear to predate the Holometabolous–Hemimetabolous split.

Two features of the opsin phylogeny are not encompassed by the three-ancient-pigment-class hypothesis derived from the physiological data. First, there is a clade of *Drosophila* Rh1 and Rh2 opsins that branches off prior to the LW opsin clade. Basal to the *Drosophila* Rh1 and Rh2 clade, is a clade of crab (*Hemigrapsus*) opsins with similar sensitivity maxima to the *Drosophila* Rh1 opsin (480 nm). Two gene duplications of a 480-nm ancestral gene coupled with two gene losses (one in insects and one in crustaceans) and a spectral shift along one branch (from 480 nm to green) could explain the observed pattern. Alternatively, an equally parsimonious scenario that involves convergent evolution could also explain the data. In this scenario, two gene duplications are coupled with one loss and two functional changes (from 480 nm to green and then a reversion from green to 480 nm). Under either scenario, the observed branching pattern suggests that some of the earliest invertebrate opsins may have had spectral properties in the blue–green (480 nm) range, with the LW (520–575 nm) opsins arising secondarily.

Despite the likelihood of this scenario, a homologue of the *Drosophila* Rh1 opsin in *P. glaucus* was not found. This is somewhat surprising because Rh1 is the pigment found in the majority of photoreceptor cells in the *Drosophila* retina, but is consistent with the lack of 480 nm-sensitive photoreceptor cells in the *Papilio* retina. Several possibilities may explain this absence. A *Drosophila* Rh1 ortholog may have become a pseudogene in the lineage leading to *Papilio.* Alternatively, its expression may be restricted to a small number of photoreceptor cells, such as the simple eyes or ocelli, which in *Papilio* are visible only under a scanning electron microscope (Dickens and Eaton 1973). Expression of a mRNA transcript in only a few cells under this scenario would be difficult to detect by the method used in this study, RT-PCR. Notably, no *Drosophila* Rh1 homologs have been isolated from any other insect species surveyed.

Origins of Red Pigments in Insects

Red-sensitive photoreceptor cells have been found in dragonflies (Yang and Osorio 1991), solitary bees, sawflies (Peitsch et al. 1992), butterflies, and moths (Bernard 1979). Of these orders, Hymenoptera, and Lepidoptera have been extensively surveyed by electrophysiology (Eguchi et al. 1982). In Hymenoptera, species with red-sensitive photoreceptor cells are relatively rare, whereas within Lepidoptera, species with red-sensitive photoreceptor cells are more common. Four out of seven insect orders—Blattaria, Orthoptera, Heteroptera, and Diptera—do not have red-sensitive photoreceptor cells, while all insect orders have green-sensitive photoreceptor cells. The sporadic appearance of the red pigments, detected through physiological and photochemical surveys of the adult insect eye, suggests that either the red pigments arose once within insects, and then there were a large number of gene losses, or that red pigments evolved more than once.

Butterflies of the genus *Papilio* are among the most well-documented examples of insect taxa with red-sensitive photoreceptor cells in addition to green-, blue-, and UV-sensitive (Horridge et al. 1983, 1984; Matic 1983; Arikawa et al. 1987). Because spectrally similar opsins cluster with one another phylogenetically (Fig. 4), it is likely that PglRh1–4 encode LW pigments, PglRh5 encodes a UV pigment, and PglRh6 encodes a blue pigment. Phylogenetic inference of the opsins' spectral properties is indirect, however, and requires independent

confirmation. Therefore, I examined the spatial expression of the *Papilio* opsins and opsin transcripts using in situ hybridization and immunohistochemistry (data not shown). Anti-PglRh5 antibody staining of the adult *P. glaucus* retina is correlated with the distribution of UV-sensitive photoreceptors; similarly PglRh6 protein expression in the retina is consistent with the distribution of blue-sensitive photoreceptor cells (Briscoe and Nagy unpublished). Kitamoto et al. (1998) find that both PxRh1 and PxRh2 mRNAs are expressed in green-sensitive photoreceptor cells, whereas PxRh3 mRNA is expressed in red-sensitive photoreceptor cells. Similar in situ hybridization experiments performed on *P. glaucus* eye slices with riboprobes to PglRh1–3 confirm their results (Briscoe and Nagy unpublished).

If there were a single origin of red pigments in insects, we would not expect the lepidopteran LW opsins (*Papilio* Rh1–4 and *M. sexta* 1) to have a monophyletic origin. Instead, the *Papilio* opsins would fall into at least two distinct clades. One of these clades would include all green insect opsins, such as *Papilio* Rh1 and Rh2, and the opsins from *Apis, Camponotus, Cataglyphis, Schistocerca,* and *Sphodromantis,* species with green-sensitive photoreceptor cells but without red-sensitive (Fig. 4). The other clade would include PglRh3 and PxRh3. Notably, this pattern is not observed. The *Papilio* opsin (Rh3) localized by in situ hybridization to red-sensitive photoreceptor cells in the retina is most closely related to a *Papilio* opsin (Rh1) localized to green-sensitive photoreceptor cells (Kitamoto et al. 1998; Briscoe and Nagy unpublished). In parsimony and distance analyses, the boot strap support for the clade composed of PglRh1–PxRh1 and PglRh3–PxRh3 opsins is high. Basal to the PglRh1–PxRh1–PglRh3–PxRh3 clade is a clade composed of green-sensitive PglRh2–PxRh2 opsins, recovered in all analyses.

Appearing basal to the clades discussed above is PglRh4, whose spatial distribution in the retina is unknown and whose clustering with the other lepidopteran LW opsins is supported by 59% of bootstrap replicates. Whether its absorption spectrum maximum is red or green, its placement in the opsin phylogeny is compatible with the hypothesis that red pigments in insects evolved more than once. Notably, the only green insect pigment to have been expressed and spectrally characterized, *D. melanogaster* Rh6 (Salcedo et al. 1999) is ancestral to all lepidopteran LW opsins (bootstrap support 85%). The particular branching pattern among the lepidopteran opsins, and their relationship to the *D. melanogaster* green pigment suggests that the red pigment in *Papilio* (PxRh3–PglRh3) evolved from a green lepidopteran ancestor. These molecular phylogenetic observations favor Chittka's (1996) hypothesis, based on physiological observations, that red pigments have evolved convergently from an ancient class of green pigments.

Spectral Tuning of the Vertebrate Red and Green Pigments and Comparisons with Papilio

Within vertebrates, gene duplication and spectral diversification of the red and green pigments has occurred independently in primates and at least twice in fish (Yokoyama and Yokoyama 1990; Johnson et al. 1993). The green fish opsins, from goldfish and from cave fish, respectively, fall into two distinct clades of vertebrate pigments. Two goldfish green pigments are most closely related to the vertebrate rod pigments, and have an average λmax value of 508 nm (Johnson et al. 1993). The goldfish (559 nm) and cave fish red pigments (Yokoyama and Yokoyama 1990; Yokoyama and Radlwimmer 1999), and two cave fish green pigments are found in a separate clade, which includes the primate red and green pigments (See Johnson et al. 1993). The green pigments in this distantly related clade have an average λmax value of 531 nm (see Yokoyama and Radlwimmer 1999 for references).

The fact that both vertebrates and insects have paralogously derived red and green pigments raises the possibility that spectral tuning mechanisms, the amino acid substitutions causing red or blue shifts in absorption spectrum maximum, may be similar. To address this question comparatively, 26 vertebrate opsin sequences were manually aligned with the invertebrate opsin sequences (data not shown), and homologous sites used in vertebrate spectral tuning were identified (Table 3). Five amino acid sites are primarily responsible for generating the spectral diversity among a variety of red and green vertebrate pigments, and the effects of substitutions at these amino acid sites are additive (Yokoyama and Radlwimmer 1999).

Three of the five critical amino acid sites (aa 180, 277, 285) involve the loss or gain of a hydroxyl group. Substitutions at these sites have some of the largest effects on spectral tuning in the red and green vertebrate pigments (Table 4). These sites are invariant in the *Papilio* LW opsins (PglRh1–4) (Table 3). A fourth vertebrate spectral tuning site (aa 197) involves a second mechanism giving rise to red shifts in vertebrate opsins—a chloride-binding site. Loss of H at this site leads to a blue shift in the mouse green pigment (Sun et al. 1997). All insect opsins, both SW and LW, have an E at aa 197, as do all SW vertebrate opsins. The observed pattern of invariance at this site in the *Papilio* LW opsins means that the chloride-binding H197 cannot be involved in spectrally diversifying the *Papilio* LW opsins.

Another spectral tuning mechanism in vertebrates involves the acquisition of a hydroxyl group near the retinylidene Schiff base. which causes a blue shift (aa A308S) (Sun et al. 1997). In *Papilio,* all SW opsins have A at aa 308, whereas all LW opsins have an S. The *Papilio* LW opsins with an S at aa 308 could be blue shifted relative to the vertebrate green pigments with an

Table 3. Nonconservative amino acid replacements between the *Papilio* LW opsins. Amino acids at homologous sites in human red (R), green (G), and Blue (B) pigments; major tuning sites in vertebrate opsins (bold and boxed)

	Papilio glaucus Rh						Human		
	LW				SW		LW		SW
AA site	1	2	3	4	5	6	R	G	B
59	V	T	T	T	V	I	W	W	F
65	F	F	I	F	A	V	T	I	L
66	I	I	T	I	A	T	A	A	I
69	S	T	T	V	I	I	F	F	P
104	T	L	A	F	L	I	V	V	I
106	**M**	**M**	**M**	**M**	**M**	**A**	**A**	**A**	**S**
107	A	A	A	S	—	—	S	S	V
136	F	F	Y	F	S	S	C	C	A
138	**T**	**S**	**T**	**C**	**T**	**I**	**I**	**I**	**L**
140	**S**	**S**	**S**	**S**	**A**	**G**	**G**	**G**	**T**
179	S	F	S	A	Y	W	W	W	I
180	**S**	**S**	**S**	**S**	**T**	**S**	**S**	**A**	**G**
197	**E**	**E**	**E**	**E**	**E**	**E**	**H**	**H**	**E**
224	A	S	A	G	F	F	M	M	F
229	F	Y	F	F	V	A	I	I	I
230	T	M	L	A	F	I	I	T	V
240	F	F	W	Y	S	S	L	L	T
277	**W**	**W**	**W**	**W**	**Y**	**F**	**Y**	**F**	**F**
285	**L**	**L**	**L**	**L**	**G**	**A**	**T**	**A**	**A**
289	F	Y	F	F	L	M	C	C	M
290	T	T	A	M	I	T	F	F	Y
303	**G**	**A**	**S**	**T**	**V**	**A**	**M**	**M**	**L**
308	**S**	**S**	**S**	**S**	**A**	**A**	**A**	**A**	**S**
311	**A**	**A**	**A**	**A**	**C**	**C**	**A**	**A**	**S**
315	**A**	**A**	**A**	**A**	**A**	**S**	**T**	**T**	**C**

Amino acids numbered relative to human red opsin sequence (Neitz et al. 1991); dashes indicate differences in opsin length. Amino acid replacements associated with blue shifts between rhodopsin and blue cone pigments: M102L, G106S, A133G, E138L, A140T, W281Y, A308S, A311S, A315C (Lin et al. 1998). Amino acids fixed in *Papilio* opsins: M102, G133, and W281

Table 4. Spectral tuning sites in vertebrates

Mutation	Effect in vertebrates	Reference
A180S	7-nm red shift	Neitz et al. (1991)
F277Y	10-nm red shift	Merbs and Nathans (1993)
A285T	16-nm red shift	Asenjo et al. (1994)
H197Y	28-nm blue shift	Sun et al. (1997)
S308A	18-nm red shift	Sun et al. (1997)

S at this position, although this may not be the case. The vertebrate green pigments with S at aa 308 include goldfish (505 and 511 nm) (Johnson et al. 1993), mouse (508 nm), and rat (509 nm) opsins (Yokoyama and Radlwimmer 1999). By contrast, the *Papilio* LW pigments have λmax values of 520, 530, and 575 nm, respectively (Arikawa et al. 1999a, 1999b). If anything, the *Papilio* opsins with S at aa 308 (Rh1–4) are 12–70 nm red shifted compared to the vertebrate pigments with the same residue at this site. Vertebrate pigments with λmax values closer to the putative *Papilio* LW pigments, which include the human red (560 nm) and green (530 nm), and squirrel (532 nm) pigments have an A at aa 308 (Yokoyama and Radlwimmer 1999).

Conceivably, an A at aa 308, if introduced into the *Papilio* LW opsins through site-directed mutagenesis, could result in absorption spectrum maxima that are red shifted relative to their present position. Such a change in peak absorption would be consistent with the mechanism in vertebrates. If this were the case, the fact that this amino acid is fixed between all SW and LW insect pigments is remarkable. It suggests that a mechanism for making long-wavelength insect pigments even longer is not being used, perhaps because of some unknown functional constraint. Even so, the S308A substitution, which is involved in generating some of the absorption spectrum maximum diversity between some of the vertebrate red and green visual pigments, is not apparently involved in spectrally diversifying the *Papilio* LW pigments.

Therefore, although there has been strong natural selection for the evolution of red and green pigments in both vertebrates and insects, some of the tuning sites of major effect used to diversify their absorption spectrum maxima are apparently different. This observation begs further investigation of spectral tuning mechanisms in the *Papilio* LW pigments. Figure 5 shows a number of nonconservative amino acid substitutions that are ripe for investigation via a *Drosophilia* transgenic system that allows the expression and spectral analysis of exogenous opsins (Feiler et al. 1992; Britt et al., 1993; Townson et al. 1998). Among those nonconservative substitutions, at least one amino acid site (aa 229) with minor effect (1–2 nm) in vertebrates (Asenjo et al. 1994; Shyue et al. 1998) may be utilized in diversifying the *Papilio* LW opsins. In a study of crayfish green opsins, Crandall and Cronin (1997) found F229Y to be one of the amino acid substitutions associated with the evolution of a 530-nm opsin from a 520-nm ancestral opsin.

In conclusion, the phylogeny of invertebrate opsins is consistent with the observation that most insects have ancient classes of UV, blue and green pigments (Chittka 1996). *Papilio* butterflies have opsins that fall within each of these major clades. Duplications of the ancestral LW opsin gene have occurred at least three times in the lineage leading to *Papilio*. The recovery of a monophyletic clade of lepidopteran LW opsins provides corroborating evidence for the paralogous origin of red pigments in insects. Different key amino acid residues are likely to be involved in the spectral diversification of the *Papilio* red and green pigments than those involved in diversifying closely related red and green vertebrate pigments.

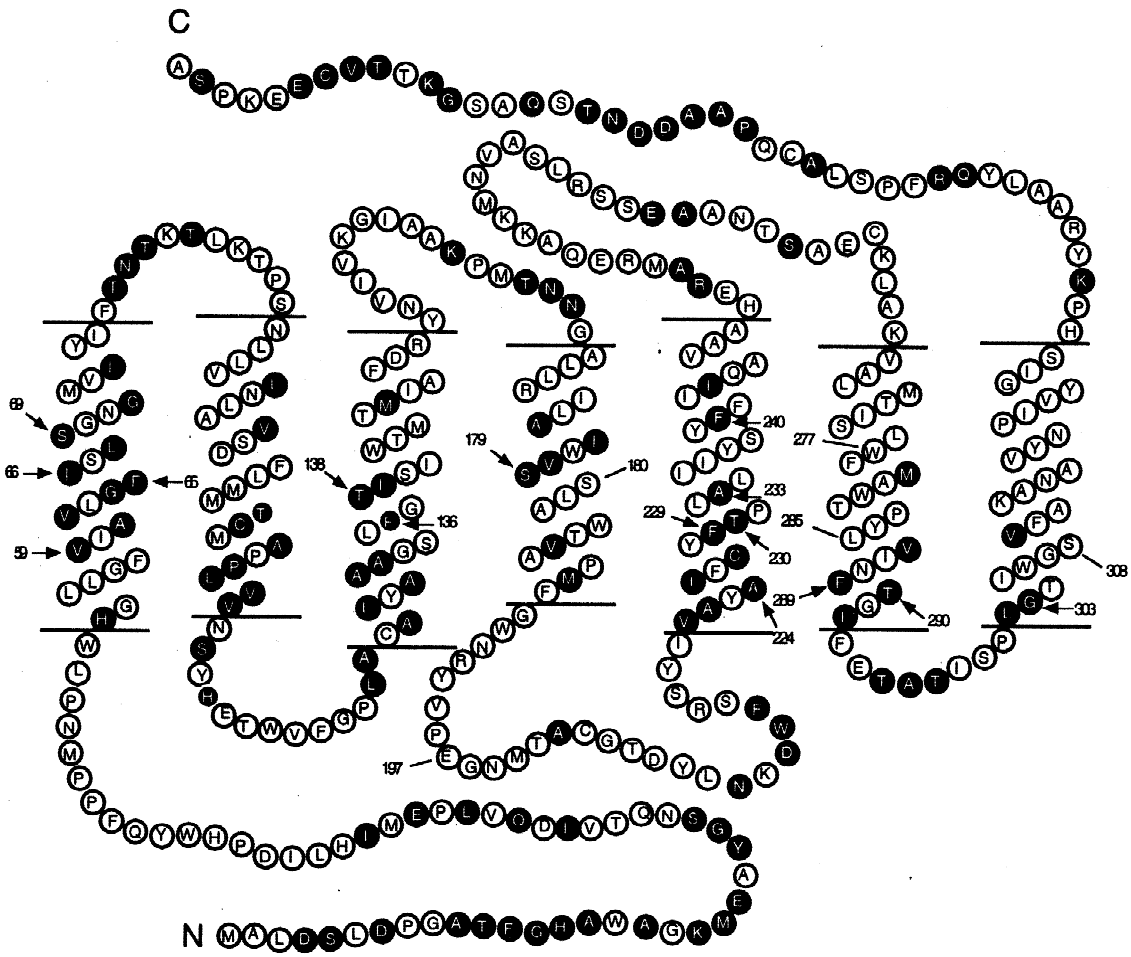

Fig. 5. Topographical model showing the amino acid differences (*filled circles*) between *Papilio glaucus* LW opsins PglRh1–4. The diagram is based on a model of *Drosophila* Rh1 from Britt et al. (1993). The amino acid sequence of PglRh1 is shown. The amino acid residues are numbered relative to the human red opsin sequence. *Black arrows:* positions where nonconservative amino acid substitutions have occurred between PglRh1–4. *Dashes:* the five vertebrate spectral tuning sites discussed in the text.

The *Papilio* LW opsins provide an example of how similar visual pigment absorption spectra can be generated through shifts in the underlying patterns of amino acid substitution.

Appendix

Accession numbers for sequences used in Fig. 1 and 2. *Crustaceans,* crayfish: *Cambarellus schufeldtii* (AF003544); *Cambarus hubrichti* (AF005385); *C. ludovicianus* (AF003543); *C. maculatus* (AF005386); *Orconectes virilis* (AF003545); *Procambarus clarkii* (S53494); *P. milleri* (AF003546); *P. orcinus* (AF005389); *P. seminolae* (AF005388); crab: *Hemigrapsus sanguineus* (1, D50583; 2, D50584). *Chelicerates:* horseshoe crab, *Limulus polyphemus* (1, L03781; 2, L03782). *Insects:* Diptera: flies, *Calliphora vicina* (Rh1, M58334); *Drosophila melanogaster* (Rh1, K02315; Rh2, M12896; Rh3, M17718; Rh4, M17730; Rh5, U67905; Rh6, Z86118); *D. pseudoobscura* (Rh1, S45126; Rh2, S45127; Rh3, S45849; Rh4, S45850); *D. subobscura* (Rh1, AF025813); *D. virilis* (Rh4, M77281); *D. mercatorum* (Rh1), *D. simulans* (Rh2), and *D. virilis* (Rh1) sequences were from (Carulli et al. 1994). Hymenoptera: bee, *Apis mellifera* (UV, AF004169; Blue, AF004168; LW, U26026); ants, *Camponotus abdominalis* (LW, U32502; SW, AF042788); *Cataglyphis bombycinus* (LW, U32501; SW, AF042787). Lepidoptera: moth, *Manduca sexta* (1, 76082; 2, 109852; 3, 1249561); butterflies, *Papilio glaucus* (PglRh1, AF077189; PglRh2, AF077190; PglRh3, AF067080; PglRh4, AF077193; PglRh5, AF077191; PglRh6, AF077192); *P. xuthus* (PxRh1, AB007423; PxRh2, AB007424; PxRh3, AB007425). Mantodea, Mantid, *Sphodromantis* (X71665). Orthoptera, Locust, *Schistocerca gregaria* (1, X80071; 2, X80072). *Molluscs:* squid, *Alloteuthis subulata* (Z49108); octopus, *Octopus dolfleini* (X07797).

Acknowledgments. I am grateful for the technical advice of B. Chang, B. Richter, K. Ober, and D. Nurminsky; the support of R.C. Lewontin and N.E. Pierce; the helpful comments of A. Berry, S. Britt, L. Chittka, R. Collins, D. Haig, E. Kellogg, R.C. Lewontin, L. Nagy, and two anonymous reviewers; and to K. Arikawa for sharing publication preprints. This work was supported by a HHMI Predoctoral Fellowship, a NSF Dissertation Improvement grant and a Sigma Xi Grant-in-Aid of Research to A.D.B, and a NIH grant to R.C. Lewontin.

References

Arikawa K, Inokuma K, Eguchi E (1987) Pentachromatic visual system in a butterfly. Naturwissenchaften 74:297–298

Arikawa K, Scholten DGW, Kinoshita M, Stavenga DG (1999a) Tun-

ing of photoreceptor spectral sensitivities by red and yellow pigments in the butterfly *Papilio xuthus.* Zool Sci 16:17–24

Arikawa K, Mizuno S, Scholten DGW, Kinoshita M, Seki T, Kitamoto J, Stavenga DG (1999b) An ultraviolet absorbing pigment causes a narrow-band violet receptor and a single-peaked green receptor in the eye of the butterfly *Papilio*. Vis Res 39:1–8

Asenjo A, Rim J, Oprian D (1994) Molecular determinants of human red/green color discrimination. Neuron 12:1131–1138

Bandai K, Arikawa K, Eguchi E (1992) Localization of spectral receptors in the ommatidium of butterfly compound eye determined by polarization sensitivity. J Comp Physiol 171:289–297

Bellingham J, Wilkie S, Morris A, Bowmaker J, Hunt D (1997) Characterization of the ultraviolet-sensitive opsin gene in the honey bee, *Apis mellifera*. Eur J Biochem 243:775–781

Bernard GD (1979) Red-absorbing visual pigments of butterflies. Science 203:1125–1127

Briscoe AD (1998) Molecular diversity of visual pigments in the butterfly *Papilio glaucus.* Naturwissenschaften 85:33–35

Briscoe AD (1999) Intron splice sites of *Papilio glaucus* PglRh3 corroborate insect opsin phylogeny. Gene 230:101–109

Britt SG, Feiler R, Kirschfeld K, Zuker CS (1993) Spectral tuning of rhodopsin and metarhodopsin in vivo. Neuron 11:29–39

Carulli JP, Chen D-M, Stark WS, Hartl DL (1994) Phylogeny and physiology of *Drosophila* opsins. J Mol Evol 38:250–262

Chittka L (1996) Does bee color vision predate the evolution of flower color? Naturwissenschaften 83:136–138

Crandall K, Cronin T (1997) The moleclar evolution of visual pigments of freshwater crayfishes (Decapoda: Cambaridae). J Mol Evol 45: 524–534

Dickens J, Eaton J (1973) External ocelli in Lepidoptera previously considered to be anocellate. Nature 242:205–206

Eguchi E, Watanabe K, Hariyama T, Yamamoto K (1982) A comparison of electrophysiologically determined spectral responses in 35 species of Lepidoptera. J Insect Physiol 28:675–682

Feiler R, Bjornson R, Kirschfeld K, Mismer D, Rubin GM, Smith DP, Socolich M, Zuker CS (1992) Ectopic expression of ultraviolet-rhodopsins in the blue photoreceptor cells of *Drosophila:* visual physiology and photochemistry of transgenic animals. J Neurosci 12:3862–3868

Felisberti F, Ventura DF (1996) Cerebral extraocular photoreceptors in ants. Tiss Cell 28:25–30

Fleissner G, Fleissner G, Frisch B (1993) A new type of putative non-visual photoreceptors in the optic lobe of beetles. Cell Tiss Res 273:435–445

Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNA from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci USA 85: 8998–9002

Gribakin FG (1988) Photoreceptor optics of the honeybee and its eye colour mutants: the effect of screening pigments on the long-wave subsystem of colour vision. J Comp Physiol A 164:123–140

Herrin DL, Schmidt GW (1988) Rapid, reversible staining of northern blots prior to hybridization. Biotechniques 6:196–200

Hofbauer A, Buchner E (1989) Does *Drosophila* have seven eyes? Naturwissenschaften 76:335–336

Horridge G, Marcelja L, Jahnke R (1984) Colour vision in butterflies. J Comp Physiol 155:529–542

Horridge G, Marcelja L, Matic T (1983) Single electrode studies on the retina of the butterfly *Papilio*. J Comp Physiol 150:271–294

Johnson RL, Grant KB, Zankel TC, Boehm MF, Merbs SL, Nathans J, Nakanishi K (1993) Cloning and expression of goldfish opsin sequences. Biochemistry 32:208–214

Kitamoto J, Sakamoto K, Ozaki K, Mishina Y, Arikawa K (1998) Two visual pigments in a single photoreceptor cell: identification and histological localization of three mRNAs encoding visual pigment opsins in the retina of the butterfly *Papilio xuthus.* J Exp Biol 201:1255–1261

Kozak M, (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. J Biol Chem 266:19867–19870

Kumar S, Koichiro T, Nei M (1993) MEGA: molecular evolutionary genetics analysis, version 1.01. Pennsylvania State University, University Park, PA

Li W-H (1997) Molecular evolution. Sinauer Associates, Sunderland, MA, pp 1–487

Lin SW, Kochendoerfer GG, Carroll KS, Wang D, Mathies RA, Sakmar TP (1998) Mechanisms of spectral tuning in blue cone pigments. J Biol Chem 273:24583–24591

Maddison DR, Baker MD, Ober KA (1999) Phylogeny of carbid beetles as inferred from 18S ribosomal DNA (Coleoptera: Carabidae). System Entomol 24:103–138

Matic T (1983) Electrical inhibition in the retina of the butterfly *Papilio*. I. Four spectral types of photoreceptors. J Comp Physiol 152:169–182

Meinertzhagen IA, Menzel R, Kahle G (1983) The identification of spectral receptor types in the retina and lamina of the dragonfly *Sympetrum rubicundulum.* J Comp Physiol 151:295–310

Merbs S, Nathans J (1993) Role of hydroxyl-bearing amino acids in differentially tuning the absorption spectra of the human red and green cone pigments. Photochem Photobiol 58:706–710

Montell C, Jones K, Zuker C, Rubin G (1987) A second opsin gene expressed in the ultraviolet-sensitive R7 photoreceptor cells of *Drosophila melanogaster*. J Neurosci 7:1558–1566

Mote MI, Goldsmith TH (1970) Spectral sensitivities of color receptors in the compound eye of the cockroach *Periplaneta.* J Exp Biol 173:137–145

Neitz M, Neitz J, Jacobs G (1991) Spectral tuning of pigments underlying red-green color vision. Science 252:971–974

Peitsch D, Feitz A, Hertel H, de Souza J, Ventura D, Menzel R (1992) The spectral input systems of hymenopteran insects and their receptor-based colour vision. J Comp Physiol 170:23–40

Rice KA (1994) The origin, evolution and classification of G protein-coupled receptors. PhD thesis, Harvard University, Cambridge, MA

Rost B, Casadio R, Fariselli P, Sander C (1995) Prediction of helical transmembrane segments at 95% accuracy. Prot Sci 4:521–533

Sakamoto K, Hisatomi O, Tokunaga F, Eguchi E (1996) Two opsins from the compound eye of the crab *Hemigrapsus sanguineus.* J Exp Biol 199:449–450

Salcedo E, Huber A, Henrich S, Chadwell LV, Chou W-H, Paulsen R, Britt SG (1999) Blue- and green-absorbing visual pigments of *Drosophila:* ectopic expression and physiological characterization of the R8 photoreceptor cell-specific Rh5 and Rh6 rhodopsins. J Neurosci 19:10716–10726

Schulz W-D, Schluter U, Seifert G (1984) Extraocular photoreceptors in the brain of *Epilachna varivestis* (Coleoptera, Coccinellidae). Cell Tiss Res 236:317–320

Shyue SK, Boissinot S, Schneider H, Sampaio I, Schneider MP, Abee CR, Williams L, Hewett-Emmett D, Sperling HG, Cowing JA, Dulai KS, Hunt DM, Li WH (1998) Molecular genetics of spectral tuning in New World monkey color vision. J Mol Evol 46:697–702

Smith W, Price D, Greenberg R, Battelle B-A (1993) Opsins from the lateral eyes and ocelli of the horseshoe crab, *Limulus polyphemus.* Proc Natl Acad Sci USA 90:6150–6154

Sun H, Macke J, Nathans J (1997) Mechanisms of spectral tuning in the mouse green cone pigment. Proc Natl Acad Sci USA 94:8860–8865

Swofford DL (1998) PAUP*4d64. Sinauer Associates, Sunderland, MA

Thompson J, Higgins D, Gibson T (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Townson SM, Chang BSW, Salcedo E, Chadwell LV, Pierce NE, Britt

SG (1998) Honeybee blue-and ultraviolet-sensitive opsins: cloning, heterologous expression in *Drosophila*, and physiological characterization. J Neurosci 18:2412–2422

Yang E-C, Osorio D (1991) Spectral sensitivities of photoreceptors and lamina monopolar cells in the dragonfly, *Hemicordulia tau*. J Comp Physiol 169:663–669

Yokoyama R, Yokoyama S (1990) Convergent evolution of the red- and green-like visual pigment genes in fish, *Astyanax fasciatus*, and human. Proc Natl Acad Sci USA 87:9315–9318

Yokoyama S, Radlwimmer B (1999) The molecular genetics of red and green color vision in mammals. Genetics 153:919–932

Zhou Y-H, Li W-H (1996) Gene conversion and natural selection in the evolution of X-linked color vision genes in higher primates. Mol Biol Evol 13:780–783

Zuker C, Mismer D, Hardy R, Rubin G (1988) Ectopic expression of a minor *Drosophila* opsin in the major photoreceptor cell class: distinguishing the role of primary receptor and cellular context. Cell 55:475–482